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(54) Title: CLONING OF FULL-LENGTH HUMAN PEX cDNA

(57) Abstract

The present invention relates to the cloning of full-length human PEX cDNA isolated from tumors causing oncogenous hypophosphatemia osteomalacia, uses of PEX active site for the design of drugs to inhibit protein activity in cases of hyperphosphatemia or chronic renal failure, uses of the PEX active site as a target for the treatment of hyperphosphatemia or chronic renal failure and uses in the diagnosis of hyperphosphatemia or chronic renal failure, use of PEX for the design of drugs to inhibit protein activity in cases of hyperphosphatemia or chronic renal failure, use of PEX as a target for the treatment of hyperphosphatemia, chronic renal failure, hypophosphatemia or idiopathic hypercalcuria, and use of PEX in the diagnosis of hyperphosphatemic states, chronic renal failure, hypophosphatemic states or idiopathic hypercalcuria.

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CLONING OF FULL-LENGTH HUMAN PEX cDNA

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to the cloning of full-length human PEX cDNA isolated from tumors causing oncogenous hypophosphatemia osteomalacia, uses of PEX active site for the design of drugs to inhibit protein activity in cases of hyperphosphatemia or chronic renal failure, uses of the PEX active site as a target for the treatment of hyperphosphatemia or chronic renal failure and uses in the diagnosis of hyperphosphatemia or chronic renal failure, use of PEX for the design of drugs to inhibit protein activity in cases of hyperphosphatemia or chronic renal failure, use of PEX as a target for the treatment of hyperphosphatemia, chronic renal failure, hypophosphatemia or idiopathic hypercalcuria, and use of PEX in the diagnosis of hyperphosphatemic states, chronic renal failure, hypophosphatemic states or idiopathic hypercalcuria.

(b) Description of Prior Art

Oncogenous hypophosphatemic osteomalacia (OHO) is a rare acquired disease characterized by severe hypophosphatemia, inappropriate phosphaturia, reduced vitamin D levels, and defective bone mineralization (Ryan, E.A. and Reiss, E., 1984, *The American Journal of Medicine*, **77**:501-512). This syndrome is associated with a variety of histologically distinct, usually benign, mesenchymal tumors. Resection of the tumor reverses the metabolic abnormalities and results in cure of the bone disease. It has been postulated that a phosphaturic factor produced by these tumors promotes the renal phosphate loss, which in turn results in osteomalacia. The putative phosphaturic factor may also

inhibit the renal conversion of 25-hydroxyvitamin D3 to 1,25-dihydroxy-vitamin D3. Depressed 1,25-dihydroxyvitaminD3 levels and chronic phosphate depletion may act synergistically to produce osteomalacia in these patients. The nature of the phosphaturic substance remains unknown and is distinct from parathyroid hormone and calcitonin, two polypeptide hormones known to inhibit the tubular reabsorption of phosphorus.

X-linked hypophosphatemia (HYP) is an inherited disorder of phosphate homeostasis with biochemical and physical findings very similar to OHO (Scriver, C.R. and Tenenhouse, H.S., 1992, *J. Inher. Metab. Dis.*, **15**:610-624). By positional cloning, a gene which spans the deleted region Xp22.1 in HYP patients, or is mutated in non-deletion patients has been identified and its partial cDNA sequence reported. This gene exhibits homology to a family of endopeptidase genes involved either in activation or degradation of a number of peptide hormones and has been named PEX (phosphate regulating gene with homologies to endopeptidases, on the **X** chromosome) (The HYP Consortium, 1995, *Nature Genetics*, **11**:130-136).

It would be highly desirable to be provided with a means to inhibit protein activity in cases of hyperphosphatemia, to be provided with a target for the treatment of hyperphosphatemia and to be provided with a diagnostic tool for hyperphosphatemic states.

SUMMARY OF THE INVENTION

One aim of the present invention is to employ the PEX active site of the design of drugs to inhibit protein activity in cases of hyperphosphatemia.

Another aim of the present invention is to employ the PEX active site as a target for the treatment of hyperphosphatemic or hypophosphatemic disorders

such as chronic renal failure, or idiopathic hypercalcuria, respectively.

Another aim of the present invention is to employ the PEX active site in the diagnosis of hyperphosphatemic and hypophosphatemic disorders.

The availability of full length PEX cDNA provides us with an unprecedented opportunity to study the biology of PEX and evaluate its role in conditions such as OHO, idiopathic hypercalcuria, HYP (a hypophosphatemic disorder) and in common pathological states characterized by impaired phosphate excretion including the large and expanding population of patients with chronic renal failure.

In accordance with the present invention there is provided a recombinant PEX protein generated from cloned cDNA depicted in Figs. 1A to 1G.

In accordance with the present invention there is provided a polyclonal or monoclonal antibody raised against the PEX active site which consists of at least amino acid residue 579 to residue 749 illustrated in Figs. 1A to 1G.

In accordance with the present invention there is provided a method for the design of drugs to be used as competitive inhibitors or activators of PEX enzymatic activity and/or its receptor in cases of hyperphosphatemia (as in chronic renal failure) or hypophosphatemia, which comprises the steps of:

a) developing a radiolabeled or fluorescent-labeled metalloendopeptidase substrate which reversibly or irreversibly binds PEX; and

b) using PEX and the labeled ligand to screen an expression library for an endogenous protein which binds PEX;

or

-using labeled PEX to examine its competitive binding to a receptor;

or

-using labeled recombinant PEX to screen an expression library in order to clone its receptor.

In accordance with the present invention there is provided a method for the treatment of hyperphosphatemia or of chronic renal failure which comprises administering to a patient an effective amount of a pharmaceutical compound targeted to inhibit PEX active site and/or its receptor.

In accordance with the present invention there is provided a method for the diagnosis of hyperphosphatemic or hypophosphatemic conditions in patient, which comprises the steps of :

a) preparing a solid support having bound thereto at least one of the anti-PEX antibody of the present invention, the recombinant PEX protein of the present invention, or the active site thereof;

b) screening a biological sample of the patient on the solid support; and

c) detecting the presence of PEX protein or PEX antibody in the sample, thereby indicating the presence of hyperphosphatemic or hypophosphatemic conditions.

In accordance with the present invention there is provided a transgenic mouse in which the wild type and mutant PEX cDNA depicted in Figs. 1A to 1G has been inserted into the murine genome to cause alterations in blood and urine phosphate and the murine counterpart of HYP and OHO. Such a transgenic mouse may be used to study the biology of PEX protein *in vivo* and its ability to reverse biochemical and physical abnormalities associated with HYP in mice and patients in the form of gene therapy.

In accordance with the present invention there is provided a method for the treatment of cancer which comprises determining the role of PEX in tumor growth by assessing its activity and/or prenylation during neoplastic transformation and using drug design to create novel anticancer treatments which interfere with PEX protein function.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1G illustrate the nucleotide sequence and predicted amino acid sequence of tumor PEX cDNA;

Figs. 2A-2C illustrate the amino acid homology between PEX and human NEP cDNA with the sequence comparison performed by LALIGN (a computer program designed to maximally align two different protein sequences); and

Fig. 3 illustrates the hydropathy plot of PEX cDNA.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, PEX expression in tumors associated with the syndrome (OHO) was examined.

We have used the PCR technique to clone and characterize full-length human PEX cDNA from two tumors associated with OHO and have determined its normal fetal and adult tissue distribution. We show that full-length human PEX cDNA encodes a 749 amino acid protein (Figs. 1A-1G) with extensive homology to the human neutral endopeptidase (Fig. 2) (NEP; EC 3.4.24.11), suggesting that PEX is a metalloendopeptidase. The additional sequences provided by our PEX cDNA clone include 603 nucleotides of the 5' noncoding region, the first 3 and the last 108 amino acids of the protein, comprising residues postulated to be critical for the formation of

the active site of the protein and hence its enzymatic activity, the termination codon, as well as 276 nucleotides of the 3' noncoding region, including the polyadenylation signal. We also show that PEX has a cleavable signal sequence and a consensus sequence for prenylation of the protein at its carboxyl terminal.

In summary, the cloning of the full-length human PEX cDNA from tumors causing OHO is reported in the present invention. The availability of this cDNA opens new and exciting avenues of investigation in a number of clinical specialties, such as endocrinology, nephrology, and oncology. The biology of PEX will be evaluated in conditions such as OHO, idiopathic hypercalcuria, and HYP.

Tumor Tissues

Tumor tissues were removed from two patients with well-documented OHO. Resection of the tumors resulted in the complete reversal of the biochemical and physical abnormalities associated with the syndrome. Tumor tissue was frozen immediately in liquid nitrogen and stored at -70°C.

PEX Expression in OHO-associated tumors

To determine whether PEX is expressed in tumors associated with OHO, RNA was extracted from tumor tissue using Qiagen RNeasy Kit™ and reversed transcribed using oligodT primer and Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was then amplified using human PEX-specific primers, PEX 1 (5' GGAGGAATTGGTTGAGGGCG 3') and PEX 2 (5' GTAGACCACCAAGGATCCAG 3'). Following amplification (35 cycles) the PCR reaction was fractionated on a 1% agarose gel stained with ethidium bromide. PEX mRNA was readily amplified from both samples demonstrating the

expected 509 bp amplified fragment, as predicted from the published partial sequence.

Cloning of full-length PEX cDNA from tumors

Cloning of the 5' end of PEX cDNA was accomplished by anchored PCR. Total cellular RNA was initially extracted from tumor tissue followed by the isolation of mRNA. 1.5 µg of mRNA was then reverse transcribed into cDNA using 200 ng of a PEX specific antisense oligomer (PEX 2) and 200 units of Superscript II (BRL) reverse transcriptase for 1 hour at 42°C in a final reaction volume of 30 µl. The resulting cDNA was size fractionated on a 1% agarose gel and fragments corresponding to >600 bp were purified and resuspended in H_2O. The 3' end of the first strand cDNA was homopolymer tailed with dGTP using 1 µl of Terminal deoxynucleotidyl transferase (TdT) at 37°C for 30 minutes in a volume of 50 µl. Following heat inactivation of the enzyme, RNA template was removed by incubation with RNase H and the tailed cDNA was purified by phenol-chloroform extraction followed by ammonium acetate precipitation. The purified tailed cDNA was resuspended in H_2O and an aliquot was used for anchored PCR along with 200 ng of an internal PEX specific antisense primer (PEX 3, 5' CGTGCCCAGAACTAGGGTGCCACC 3') and 200 ng of oligodC as the sense primer. Forty cycles of PCR were performed using 0.5 µl of Taq polymerase (Promega) in a reaction volume of 50 µl. Cycling parameters were: 1 minute of denaturation at 95°C, 2 minutes of annealing at 55°C and 2 minutes of extension at 72°C. The PCR products were fractionated on a 1% agarose gel and a band of 700 bp was isolated, purified, and ligated into pPCRII vector (Invitrogen). Following transformation into HB101 bacteria, clones containing the appropriate size insert were sequenced using Sequenase kit (US Biochem).

To clone the 3' end of PEX cDNA, an aliquot of an amplified unidirectional cDNA library in pCDNA3 vector (Invitrogen) generated from tumor mRNA was grown overnight in LB medium and plasmid DNA extracted. DNA (0.5 µg) was subjected to PCR using a PEX-specific sense oligomer (PEX1) and an antisense oligomer corresponding to SP6 RNA polymerase binding site sequences present in the pCDNA3 vector. Thirty five cycles of amplification were performed in a 50 µl reaction volume with each cycle consisting of 1 min. denaturation at 94°C, 1 min. annealing at 55°C and 1 min. extension at 72°C. Amplified products were fractionated on a 1% agarose gel and a 1.2 kb fragment corresponding to the 3' end of PEX cDNA was subcloned and sequenced.

Figs. 1A-1G show the nucleotide and predicted amino acid sequence of the full-length PEX cDNA cloned from tumor tissue. In both tumors, there were three amino acids that differ from the published partial PEX sequence, $^{363}D\text{->}A$(GAC to GCC), $^{403}R\text{->}W$(AGG to TGG), and $^{641}A\text{->}G$(GCG to GGA). Full-length human PEX cDNA encodes 749 amino acids and has extensive homology to the human neutral endopeptidase (Fig. 2) (NEP; EC 3.4.24.11), suggesting that PEX is a metalloendopeptidase. The additional sequences provided by our PEX cDNA clone include 603 nucleotides of the 5' noncoding region, as well as the first 3 and the last 108 amino acids of the protein. These additional amino acids comprise residues that may be critical for the formation of the active site of the protein and hence its enzymatic activity, such as ^{642}E, ^{710}H, and $^{693,733,746}C$. Our PEX clone also identifies the termination codon, as well as 276 nucleotides of the 3' noncoding region, including the polyadenylation signal. Hydropathy plot and PSORT analysis of the PEX protein identified a putative cleavable signal sequence composed of the first 49

amino acids, implicating amino acid 50 at the beginning of the mature protein (Fig. 3). This contrasts with the human NEP sequence that does not have a cleavable signal sequence. PEX protein has also been shown to have a carboxyl terminal motif (CAAX box: CRLW) that may direct prenylation of the protein, a post-translational modification that may be important in neoplastic processes, and could be targeted for pharmacological manipulation.

Northern-blot analysis

Total RNA was prepared from human Saos-2 osteosarcoma cells by Trizol™ and polyA+ RNA was isolated using standard procedures. Twenty micrograms of PolyA+ RNA were fractionated on 1% denaturing agarose gel, transferred to nylon membrane and probed with ^{32}P-labeled human PEX cDNA. The blot was washed in 0.1 X SSC at 55°C for 20 min., and subjected to autoradiography for 7 days. To monitor loading, the membrane was re-probed with an GAPDH cDNA probe. In these cells, a single weak transcript was detected with mobility slightly faster than 28S RNA, consistent with the predicted size from the cloned PEX cDNA (~3.1 kb) and a more intense signal was observed of ~6.6 kb size.

Tissue Distribution of PEX mRNA

Recent studies have not documented the presence of PEX mRNA in normal tissues except for fetal brain and human leukocytes (The HYP Consortium, 1995, *Nature Genetics*, 11:130-136). Following the cloning of full-length PEX, we examined PEX expression in normal fetal and adult tissues and in a number of established human cell lines using RT-PCR. PEX was found to be expressed in fetal calvarium (bone) and to a lesser degree in fetal kidney and muscle while no expression was apparent in fetal liver. In adult tissues, PEX mRNA was identified in kidney, but not in liver, or endomyocar-

dium. Interestingly, weak expression was evident in tissue from an atrial myxoma, a tumor of mesenchymal origin and in renal cell carcinoma, an epithelial tumor. A number of established human cell lines were also shown to express PEX, including the Saos-2 osteosarcoma cells.

In vitro transcription and translation

Plasmid pPEX was linearized at the XhoI site of the polylinker region and sense RNA strand was transcribed using T7 RNA polymerase. Translation reactions in rabbit reticulocyte lysate were performed according to the manufacturer's (Promega) procedure either in the absence or presence of canine pancreas microsomal membranes. Samples were processed for SDS-polyacrylamide gel electrophoretic (PAGE) analysis of the peptides, and autoradiography were performed. In the absence of microsomal membranes, PEX cRNA was translated into a ~82kD protein. Following the addition of microsomal membranes, two translated products of higher molecular weight were apparent, consistent with N-glycosylation of PEX (nine potential sites).

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXAMPLE I

Uses of recombinant PEX protein

Recombinant PEX protein will be generated from the cloned cDNA and an assay will be developed to clone the PEX substrate and/or PEX receptor which may also have important biological functions. For this assay, a soluble form of PEX protein will be used to bind a fluorescent substrate and conditioned media from COS cells transfected with various cDNA expression libraries will be used to compete with the substrate for the

PEX protein. Step-wise analysis will lead to identification of the cDNA encoding the physiological substrate of PEX. Other studies will determine if radiolabeled recombinant PEX interacts with a specific receptor and if so, the receptor will be cloned by expression cloning.

EXAMPLE II
Uses of specific anti-PEX antibody

Specific PEX antibodies will be generated for developing assays that will measure circulating levels of this peptide in various clinical states such as chronic renal failure.

EXAMPLE III
Competitive inhibitors or activators of PEX enzymatic activity

Other studies will concentrate on the role of PEX in common pathological states characterized by impaired phosphate excretion, as in patients with chronic renal failure. These patients develop hyperphosphatemia that causes a number of complications such as ectopic calcifications, secondary hyperparathyroidism and inevitable metabolic bone disease leading to increased morbidity and mortality. The potential therapeutic value of pharmacological manipulation of PEX in this condition will be examined by designing competitive inhibitors or activators of its enzymatic activity and/or its receptor, and studying their effects, first in animal models of chronic renal failure, and eventually in patients.

EXAMPLE IV
Gene transfer using PEX cDNA

In animal studies, we will use the technique of gene transfer to introduce normal and mutated PEX cDNA in normal mice and in mice with the murine counterpart of HYP to study the biology of the protein and its

ability to reverse the biochemical and physical abnormalities associated with the latter disorder. Potentially, these experiments can be extended for therapeutic purposes to patients with HYP as well as other disorders of phosphate homeostasis.

EXAMPLE V
Role of PEX in tumors

Finally, studies will also be directed in defining the role of PEX in tumor growth by assessing its activity and/or prenylation during neoplastic transformation. Prenylation is necessary for association with the plasma membrane and cell transformation. The critical role of prenylation can be exploited by the use of rational drug design to create novel anticancer treatments that interfere with PEX protein function.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. Recombinant PEX protein generated from cloned cDNA depicted in Figs. 1A to 1G.

2. A polyclonal or monoclonal antibody raised against the PEX active site which consists of at least amino acid residue 579 to residue 749 illustrated in Figs. 1A to 1G.

3. A method for the design of drugs to be used as competitive inhibitors or activators of PEX enzymatic activity and/or its receptor in cases of hyperphosphatemia (as in chronic renal failure) or hypophosphatemia, which comprises the steps of:

a) developing a radiolabeled or fluorescent-labeled metalloendopeptidase substrate which reversibly or irreversibly binds PEX; and

b) using PEX and said labeled ligand to screen an expression library for an endogenous protein which binds PEX;

or

using labeled PEX to examine its competitive binding to a receptor;

or

using labeled recombinant PEX of claim 1 to screen an expression library in order to clone its receptor.

4. A method for the treatment of hyperphosphatemia or of chronic renal failure which comprises administering to a patient an effective amount of a pharmaceutical compound targeted to inhibit PEX active site and/or its receptor.

5. A method for the diagnosis of hyperphosphatemic or hypophosphatemic conditions in patient, which comprises the steps of :

a) preparing a solid support having bound thereto at least one of the antibody of claim 2, the recombinant PEX protein of claim 1, or the active site thereof;

b) screening a biological sample of the patient on said solid support; and

c) detecting the presence of PEX protein or PEX antibody in said sample, thereby indicating the presence of hyperphosphatemic or hypophosphatemic conditions.

6. A transgenic mouse in which the wild type and mutant PEX cDNA depicted in Figs. 1A to 1G has been inserted into the murine genome to cause alterations in blood and urine phosphate in the murine counterpart of HYP and OHO.

7. The use of the mouse of claim 6 to study the biology of PEX protein *in vivo* and its ability to reverse biochemical and physical abnormalities associated with HYP in mice and patients in the form of gene therapy.

8. A method for the treatment of cancer which comprises determining the role of PEX in tumor growth by assessing its activity and/or prenylation during neoplastic transformation and using drug design to create novel anticancer treatments which interfere with PEX protein function.

```
1                                                   31
GAT CCA CTA GTA ACG GCC GCC AGT GTG GTG GAA TTC AAG GGA CTC ACA CAC TGA AAG AAT
61                                                  91
ATC TTT GAT GAA GAC AAT TCA GGC AAG CAG AAT GAT TCT TGC AAC AGA ATT ACA TGA TTA
121                                                 151
ATT GAG ATC TTG AAG TGG GTC CGG TGA ATC CTG GCC ACC TAA CTT ATC ATG ATT TGG GGG
181                                                 211
AGT TTC ACG AGA ATC CAG TTT TGA TAA AAC AAT TGT TTT TTT CCT CCC CAA GTG ACT ATA
241                                                 271
CAT TTA AAT AGC TAA AAC ATC TGT TCA GCA ACA TAG TAA AAC ATA TAT ACT CGG AAC GCT
301                                                 331
TGA GAG AAG AGC CTG CCA AAC AGG GAC TTT GCT GAG GGA GAG CAC CAA GAT AAA GCA ACA
361                                                 391
CTG TTT GTT TTG TCT AGT CAG GGG GGA AAG CCA AGG CAA CCA ATA TTT TGG TTT TTA TAA
421                                                 451
TTT TCA TTT GTG AAG AAT TAT TTG AGA AAG GGT TGG CGA GGG GAG ATT TCC TGA CGG CAG
481                                                 511
TTT CTT AAG CTG TCC ATT AGT AGA AGA GCA AGA GAG CCT TGG ATG TCA ACG CCT CGC TCT
541                                                 571
TGA GAC CAG CCA CCA AAC CAC GAA AAG TGA CTT TCT TCT CGT GTG CTC TCT ACG GCC CTT
601 /1                                              631/10
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Fig 1A

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CTG ATG GAA GCA GAA ACA GGG AGC AGC GTG GAG ACT GGA AAG AAG GCC AAC AGA GGC ACT
    M   E   A   E   T   G   S   S   V   E   T   G   K   K   A   N   R   G   T
661/20                                          691/30
CGA ATT GCC CTG GTC GTG TTT GTC GGT GGC ACC CTA GTT CTG GGC ACG ATC CTC TTT CTA
R   I   A   L   V   V   F   V   G   G   T   L   V   L   G   T   I   L   F   L
721/40                                          751/50
GTG AGT CAA GGT CTC TTA AGT CTC CAA GCT AAA CAG GAG TAC TGC CTG AAG CCA GAA TGC
V   S   Q   G   L   L   S   L   Q   A   K   Q   E   Y   C   L   K   P   E   C
781/60                                          811/70
ATC GAA GCG GCT GCT GCC ATC TTA AGT AAA GTA AAT CTG TCT GTG GAT CCT TGT GAT AAT
I   E   A   A   A   A   I   L   S   K   V   N   L   S   V   D   P   C   D   N
841/80                                          871/90
TTC TTC CGG TTC GCT TGT GAT GGC TGG ATA AGC AAT AAT CCA ATT CCC GAA GAT ATG CCA
F   F   R   F   A   C   D   G   W   I   S   N   N   P   I   P   E   D   M   P
901/100                                         931/110
AGC TAT GGG GTT TAT CCT TGG CTG AGA CAT AAT GTT GAC CTC AAG TTG AAG GAA CTT TTG
S   Y   G   V   Y   P   W   L   R   H   N   V   D   L   K   L   K   E   L   L
961/120                                         991/130
GAG AAA TCA ATC AGT AGA AGG CGG GAC ACC GAA GCC ATA CAG AAA GCC AAA ATC CTT TAT
E   K   S   I   S   R   R   R   D   T   E   A   I   Q   K   A   K   I   L   Y
1021/140                                        1051/150
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Fig - 1B

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TCA TCC TGC ATG AAT GAG AAA GCG ATT GAA AAA GCA GAT GGC AAG CCA CTG CTA CAC ATC
S   S   C   M   N   E   K   A   I   E   K   A   D   G   K   P   L   L   H   I
1081/160                                    1111/170
CTA CGG CAT TCA CCT TTC CGC TGG CCC GTG CTT GAA TCT AAT ATT GGC CCT GAA GGG GTT
L   R   H   S   P   F   R   W   P   V   L   E   S   N   I   G   P   E   G   V
1141/180                                    1171/190
TGG TCA GAG AGA AAG TTC AGC CTT CTG CAG ACA CTT GCA ACG TTT CGT GGT CAA TAC AGC
W   S   E   R   K   F   S   L   L   Q   T   L   A   T   F   R   G   Q   Y   S
1201/200                                    1231/210
AAT TCT GTG TTC ATC CGT TTG TAT GTG TCC CCT GAT GAC AAA GCA TCC AAT GAA CAT ATC
N   S   V   F   I   R   L   Y   V   S   P   D   D   K   A   S   N   E   H   I
1261/220                                    1291/230
TTG AAG CTG GAC CAA GCA ACA CTC TCC CTG GCC GTG AGG GAA GAC TAC CTT GAT AAC AGT
L   K   L   D   Q   A   T   L   S   L   A   V   R   E   D   Y   L   D   N   S
1321/240                                    1351/250
ACA GAA GCC AAG TCT TAT CGG GAT GCC CTT TAC AAG TTC ATG GTG GAT ACT GCC GTG CTT
T   E   A   K   S   Y   R   D   A   L   Y   K   F   M   V   D   T   A   V   L
1381/260                                    1411/270
TTA GGA GCT AAC AGT TCC AGA GCA GAG CAT GAC ATG AAG TCA GTG CTC AGA TTG GAA ATT
L   G   A   N   S   S   R   A   E   H   D   M   K   S   V   L   R   L   E   I
1441/280                                    1471/290
```

Fig. 1C

```
AAG ATA GCT GAG ATA ATG ATT CCA CAT GAA AAC CGA ACC AGC GAG GCC ATG TAC AAC AAA
K   I   A   E   I   M   I   P   H   E   N   R   T   S   E   A   M   Y   N   K
1501/300                                    1531/310
ATG AAC ATT TCT GAA CTG AGT GCT ATG ATT CCC CAG TTC GAC TGG CTG GGC TAC ATC AAG
M   N   I   S   E   L   S   A   M   I   P   Q   F   D   W   L   G   Y   I   K
1561/320                                    1591/330
AAG GTC ATT GAC ACC AGA CTC TAC CCC CAT CTG AAA GAC ATC AGC CCC TCC GAG AAT GTG
K   V   I   D   T   R   L   Y   P   H   L   K   D   I   S   P   S   E   N   V
1621/340                                    1651/350
GTG GTC CGC GTC CCG CAG TAC TTT AAA GAT TTG TTT AGG ATA TTA GGG TCT GAG AGA AAG
V   V   R   V   P   Q   Y   F   K   D   L   F   R   I   L   G   S   E   R   K
1681/360                                    1711/370
AAG ACC ATT GAC AAC TAT TTG GTG TGG AGA ATG GTT TAT TCC AGA ATT CCA AAC CTT AGC
K   T   I   D   N   Y   L   V   W   R   M   V   Y   S   R   I   P   N   L   S
1741/380                                    1771/390
AGG CGC TTT CAG TAT AGA TGG CTG GAA TTC TCA AGG GTA ATC CAG GGG ACC ACA ACT TTG
R   R   F   Q   Y   R   W   L   E   F   S   R   V   I   Q   G   T   T   T   L
1801/400                                    1831/410
CTG CCT CAA AGG GAC AAA TGT GTA AAC TTT ATT GAA AGT GCC CTC CCT TAT GTT GTT GGA
L   P   Q   R   D   K   C   V   N   F   I   E   S   A   L   P   Y   V   V   G
1861/420                                    1891/430
```

FIG - 10

```
AAG ATG TTT GTA GAT GTG TAC TTC CAG GAA GAT AAG AAG GAA ATG ATG GAG GAA TTG GTT
K   M   F   V   D   V   Y   F   Q   E   D   K   K   E   M   M   E   E   L   V
1921/440                                        1951/450
GAG GGC GTT CGC TGG GCC TTT ATT GAC ATG CTA GAG AAA GAA AAT GAG TGG ATG GAT GCA
E   G   V   R   W   A   F   I   D   M   L   E   K   E   N   E   W   M   D   A
1981/460                                        2011/470
GGA ACG AAA AGG AAA GCC AAA GAA AAG GCG AGA GCT GTT TTG GCA AAA GTT GGC TAT CCA
G   T   K   R   K   A   K   E   K   A   R   A   V   L   A   K   V   G   Y   P
2041/480                                        2071/490
GAG TTT ATA ATG AAT GAT ACT CAT GTT AAT GAA GAC CTC AAA GCT ATC AAG TTT TCA GAA
E   F   I   M   N   D   T   H   V   N   E   D   L   K   A   I   K   F   S   E
2101/500                                        2131/510
GCC GAC TAC TTT GGC AAC GTC CTA CAA ACT CGC AAG TAT TTA GCA CAG TCT GAT TTC TTC
A   D   Y   F   G   N   V   L   Q   T   R   K   Y   L   A   Q   S   D   F   F
2161/520                                        2191/530
TGG CTA AGA AAA GCC GTT CCA AAA ACA GAG TGG TTT ACA AAT CCG ACG ACT GTC AAT GCC
W   L   R   K   A   V   P   K   T   E   W   F   T   N   P   T   T   V   N   A
2221/540                                        2251/550
TTC TAC AGT GCA TCC ACC AAC CAG ATC CGA TTT CCA GCA GGA GAG CTC CAG AAG CCT TTC
F   Y   S   A   S   T   N   Q   I   R   F   P   A   G   E   L   Q   K   P   F
2281/560                                        2311/570
```

FIG - 1E

```
TTT TGG GGA ACA GAA TAT CCT CGA TCT CTG AGT TAT GGT GCT ATA GGA GTA ATT GTC GGA
F   W   G   T   E   Y   P   R   S   L   S   Y   G   A   I   G   V   I   V   G
2341/580                                    2371/590
CAT GAA TTT ACA CAT GGA TTT GAT AAT AAT GGT AGA AAA TAT GAT AAA AAT GGA AAC CTG
H   E   F   T   H   G   F   D   N   N   G   R   K   Y   D   K   N   G   N   L
2401/600                                    2431/610
GAT CCT TGG TGG TCT ACT GAA TCA GAA GAA AAG TTT AAG GAA AAA ACA AAA TGC ATG ATT
D   P   W   W   S   T   E   S   E   E   K   F   K   E   K   T   K   C   M   I
2461/620                                    2491/630
AAC CAG TAT AGC AAC TAT TAT TGG AAG AAA GCT GGC TTA AAT GTC AAG GGG AAG AGG ACC
N   Q   Y   S   N   Y   Y   W   K   K   A   G   L   N   V   K   G   K   R   T
2521/640                                    2551/650
CTG GGA GAA AAT ATT GCT GAT AAT GGA GGC CTG CGG GAA GCT TTT AGG GCT TAC AGG AAA
L   G   E   N   I   A   D   N   G   G   L   R   E   A   F   R   A   Y   R   K
2581/660                                    2611/670
TGG ATA AAT GAC AGA AGG CAG GGA CTT GAG GAG CCT CTT CTA CCA GGC ATC ACA TTC ACC
W   I   N   D   R   R   Q   G   L   E   E   P   L   L   P   G   I   T   F   T
2641/680                                    2671/690
AAC AAC CAG CTC TTC TTC CTG AGT TAT GCT CAT GTG AGG TGC AAT TCC TAC AGA CCA GAA
N   N   Q   L   F   F   L   S   Y   A   H   V   R   C   N   S   Y   R   P   E
2701/700                                    2731/710
```

FIG - 1F

```
GCT GCC CGA GAA CAA GTC CAA ATT GGT GCT CAC AGT CCC CCT CAG TTT AGG GTC AAT GGT
 A   A   R   E   Q   V   Q   I   G   A   H   S   P   P   Q   F   R   V   N   G
2761/720                                2791/730
GCA ATT AGT AAC TTT GAA GAA TTC CAG AAA GCT TTT AAC TGT CCA CCC AAT TCC ACG ATG
 A   I   S   N   F   E   E   F   Q   K   A   F   N   C   P   P   N   S   T   M
2821/740                                2851
AAC AGA GGC ATG GAC TCC TGC CGA CTC TGG TAG CTG GGA CGC TGG TTT ATG GCA TCC TGA
 N   R   G   M   D   S   C   R   L   W   *
2881                                    2911
GAC AGT TGC ACA GTG CCA GCG GAG GCT GCA CTG AGC CTT CAT CGC CCA TTG CTT TAG GCC
2941                                    2971
TGG AGG AGC TTT CAT TTT TAG TGC ATT TTC ATT ATT TGG GTA GGT GAC CTG CTT GGA TCT
3001                                    3031
AGA CAG CAT CTG TTC AAA GTT GTA GGG CTT ATA AAG TGG AAT ATA AGA AGA ACT AAG TAT
3061                                    3091
GTT TCT TTA GAA AAT CAA ACC AAC AAA AAT AAA TCC CTA GGC TAC TTT TGT TAA AAA AAA
3121
 AAA AAA AAA A
```

Fig - 16

```
hPEX   MEAETGSSVETGKKANRGTRIALVVFVGGTLVLGTILFLVSQGLLSLQAKQEYCLKPECI
       :::.....  .  :...  :  .....  .  :::  ...  :.   :.     .  :  ..::
hNEP   MESQMDITDINTPKPKKKQRWTPLEISLSVLVLLLTIIAVTMIALYATYDDGICKSSDCI

hPEX   EAAAAILSKVNLSVDPCDNFFRFACDGWISNNPIPEDMPSYGVYPWLRHNVDLKLKELLE
       ..::  ..  ...  .  .::  .:::.:::::.  :  :::  ..::  .  ::.  ...  :::.:.
hNEP   KSAARLIQNMDATTEPCTDFFKYACGGWLKRNVIPETSSRYGNFDILRDELEVVLKDVLQ

hPEX   KSISRRRDTEAIQKAKILYSSCMNEKAIEKADAKPLLHILRHSPFRWPVLESNIGPEGVW
       ....   :..:::::  :::::::  ::.  ..:::  .:...  ..:::   :  ...  .
hNEP   EPKT--EDIVAVQKAKALYRSCINESAIDSRGGEPLLKLLPDI-YGWPVATENWEQKYGA

hPEX   SERKFSLLQTLATFRGQYSNSVFIRLYVSPDDKASNEHILKLDQATLSLAVREDYLDNST
       :    .  ...:  .  ..:..  :.:  :.:.:::  :  :..  .:::.:.:.  :..:   ...
hNEP   SW---TAEKAIAQLNSKYGKKVLINLFVGTDDKNSVNHVIHIDQPRLGLPSRDYYECTGI

hPEX   EAKSYRDALY-KFMVDTAVLLGANSSRAEH----DMKSVLRLEIKIAEIMIPHENRT-SE
         .....  ..  ..  :..  .....  ....:.     .:.  :.  :::.::   .  .:.:.  .
```

FIG 2A

```
hNEP   YKEACTAYVDFMISVARLIRQEERLPIDENQLALEMNKVMELEKEIANATAKPEDRNDPM

hPEX   AMYNKMNISEL-SAMIPQFDWLGYIK-KVIDTRLYPHLKDISPSENVVVRVPQYFKDLFR
        .:::::.....  .   ......  ..   ....  ....   . :.   :.:::  .:.:..  :  .
hNEP   LLYNKMTLAQIQNNFSLEINGKPFSWLNFTNEIMSTVNISITNEEDVVVYAPEYLTKLKP

hPEX   ILGSERKKTIANYLVWRMVYSRIPNLSRRFQYRWLEFSRVIQGTTTLLPQWDKCVNFIES
       ::    .  .  .  :  .  ::  .   ...:::...  ..  .:....  :::.    .  :  .:.:...  .
hNEP   ILTKYSARDLQNLMSWRFIMDLVSSLSRTYKESRNAFRKALYGTTSETATWRRCANYVNG

hPEX   ALPYVVGKMFVDVYFQEDKKEMMEELVEGVRWAFIDMLEKENEWMDAGTKRKAKEKARAV
        .  ..::....:..  :  ..  :  ..:.:..  .:  .::  .:.  .    :::::.::..:.::::.:.
hNEP   NMENAVGRLYVEAAFAGESKHVVEDLIAQIREVFIQTLD-DLTWMDAETKKRAEEKALAI

hPEX   LAKVGYPEFIM-NDTHVNEDLKAIKFSEADYFGNVLQTRKYLAQSDFFWLRKAVPKTEWF
        ...:::.  :.  ::.  .:  .    ....  :..::.:..:..:..         .      ::.  :   :   ::.
hNEP   KERIGYPDDIVSNDNKLNNEYLELNYKEDEYFENIIQNLKFSQSKQLKKLREKVDKDEWI

hPEX   TNPTTVNAFYSASTNQIRFPAGELQKPFFWGTEYPRSLSYGAIGVIVGHEFTHGFDNNGR
```

FIG. 2B

```
        .  ..  ::::::..::: :::: :: :::..  ..   ::.::.::..:::.:::::.:::
hNEP    SGAAVVNAFYSSGRNQIVFPAGILQPPFFSAQQSN-SLNYGGIGMVIGHEITHGFDDNGR

hPEX    KYDKNGNLDPWWSTESEEKFKEKTKCMINQYSNYYWKKAG-LNVKGKRTLGENIADNGGL
        ...:.:.:. ::. .:. .:::..:::.:::.:..:  ::  ....:. :::::::::::::
hNEP    NFNKDGDLVDWWTQQSASNFKEQSQCMVYQYGNFSWDLAGGQHLNGINTLGENIADNGGL

hPEX    REAFRAYRKWINDRRQGLEEPLLPGITFTNNQLFFLSYAHVRCNSYRPEAAREQVQIGAH
        ..:.:::.   :....  :: ::::. ...::::::.:.:.: .:::: :    .....:
hNEP    GQAYRAYQ---NYIKKNGEEKLLPGLDLNHKQLFFLNFAQVWCGTYRPEYAVNSIKTDVH

hPEX    SPPQFRVNGAISNFEEFQKAFNCPPNSTMNRGMDSCRLW
        ::  ::..:.. : .:: .::.:. :: ::...  ::.:
hNEP    SPGNFRIIGTLQNSAEFSEAFHCRKNSYMNPEKK-CRVW
```

FIG. 2C

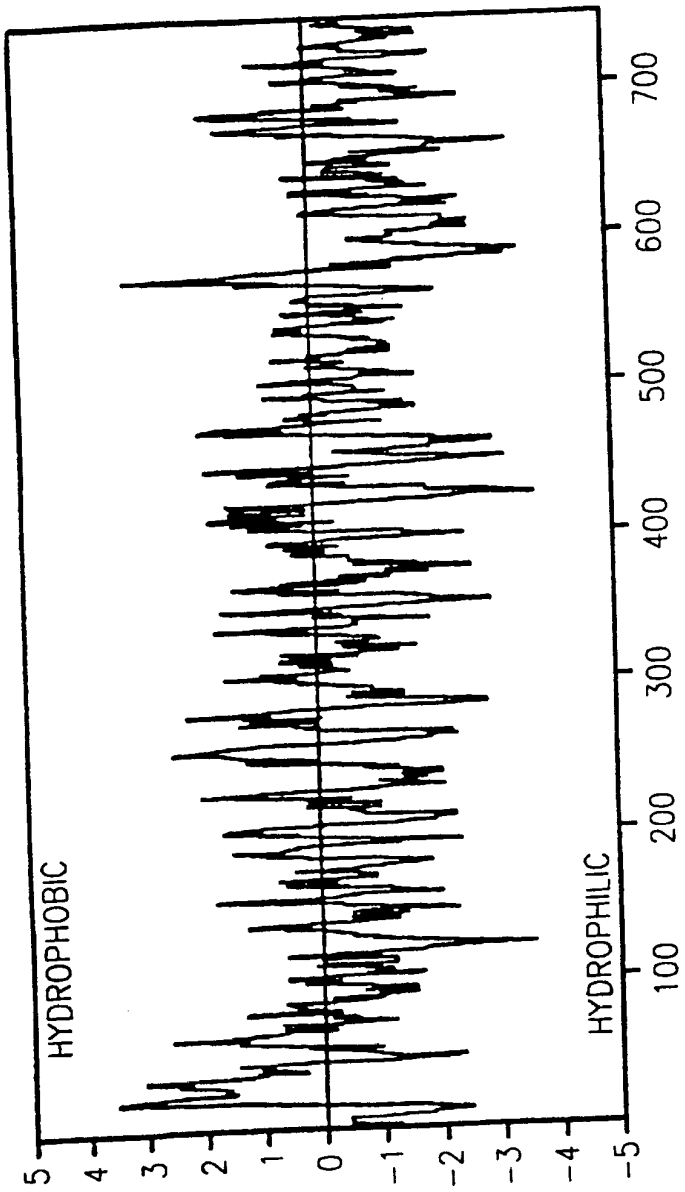
HYDROPHOBIC
HYDROPHILIC
5
4
3
2
1
0
-1
-2
-3
-4
-5
100
200
300
400
500
600
700

Fig - 3